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SEVERE DISEASE OF MELON IN NORTH WEST FRONTIER PROVINCE IS ASSOCIATED WITH SIMULTANEOUS INFECTION OF TWO RNA VIRUSES

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Abstract

During a search for cucurbit viruses in NWFP, a very severe disease of melon (*Cucumis melo* L.) characterized by leaf curling, chlorotic spots, vein clearing, mosaic, leaf distortion and enations on the upper leaf surface was identified in commercial fields in Mardan District, NWFP. Symptomatic plants were screened for the presence of *Zucchini yellow mosaic virus* (ZYMV) and *Cucumber mosaic virus* (CMV) by double antibody sandwich enzyme-linked immounosorbent assay (DAS-ELISA). Plants were also tested for the presence of begomoviruses by polymerase chain reaction and Southern hybridization using general probes. No sample was found positive for begomoviruses. The presence of CMV and ZYMV was confirmed by DAS-ELISA. Our results show that the severe disease found on melon in Mardan district is caused by multiple infections of two viruses and the severity result from synergism between two viruses.

Introduction

Cucurbits are major food crops in Pakistan. The area occupied by cucurbits in 1999-2000 was 1796 ha with an estimated production of 17256 tons (Anon., 2001). Cucurbit viruses cause significant losses to crops and continue to be a threat for future production. Several viruses belonging to potyvirus, cucumovirus and geminivirus families have been reported on cucurbits worldwide. Almost 35 different viruses have been reported to infect *Cucurbitaceae* family (Provvidenti, 1996). An interesting feature of these viruses is that, these are often found in multiple infections.

Some plant viruses interact with each other producing a worse disease than caused by either virus alone. The phenomena are termed as synergism and often result in increased accumulation of viruses involved (Sano & Kojima, 1989; Pruss *et al*., 1997). Thus, multiple infections of viruses in plants are a common phenomenon (Kassanis, 1963; Falk & Bruening, 1994). There are considerable opportunities for synergistic interactions leading to enhanced pathology in field infected plants. Combinations of such viruses leading to synergy including the potyviruses and other viruses belonging to different families are described on cucurbits and other hosts (Damirdagh & Ross, 1967; Rochow, & Ross, 1955; Vance, 1991; Vance *et a*l., 1995). Infections of cucurbit species by either potyviruses such as *Zucchini yellow mosaic virus* (ZYMV) or *Watermelon mosaic virus* (WMV) or by Cucumber mosaic virus (CMV) are very common and cause considerable damage worldwide in severe epidemics in cucurbit fields, either in single or double infections (Grafton *et al*, 1996, Luis-Arteaga *et al*, 1998). The genome organizations of CMV and potyviruses are different from each other. CMV contains a tripartite, positive sense RNA genome expressing 5 genes: 3 from the genomic RNAs 1 to 3 and 2 from subgenomic RNAs 4 and 4A (Ding *et al.*, 1994; Palukaitis *et al.*, 1992).

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Different strains of CMV can also support satellite RNAs, which attenuate CMV induced symptoms (Garcia *et al.*, 1999). Potyviruses such as ZYMV and WMV are positive sense RNA viruses and contain monopartite genome which encode a single polyprotein that is cleaved to 9 or 10 mature proteins (Revers, *et al.*, 1999). Any individual potyvirus has limited host range, but the collective host range of the large number of known potyviruses is very broad (Shukla, *et al.*, 1994). Different strains of CMV have a collective host range of over 1,000 species (Palukaitis *et al.*, 1992).

Field surveys in Pakistan often show disease symptoms on several cucurbit crops. During a search for cucurbits viruses in NWFP, a very severe disease of melon (*Cucumis melo* L.), characterized by leaf yellowing, leaf curling, chlorotic spots, vein clearing, mosaic, leaf distortion and enations on the upper leaf surface was identified in commercial fields. Here we report that this severe disease is caused by multiple infections of CMV and ZYMV.

Materials and Methods

Collection of samples: During a search for cucurbit viruses in May 2004, samples were collected from four different fields in Mardan, NWFP. At each location melon fields were visited and samples were collected moving diagonally across the field from plant showing infection. Each sample consisting of 3 leaves per plant was wrapped in polythene bags and placed in an icebox. Healthy leaves samples were taken as control. Samples were brought to Molecular Virology Laboratory of National Institute for Biotechnology and Genetic Engineering, Faisalabad.

Serology: On the basis of symptoms cucurbit-infecting viruses were suspected. Samples were screened for the presence of *Zucchini yellow mosaic virus* (ZYMV; Desbiez & Lecoq, 1997) and *Cucumber mosaic virus* (CMV) by DAS-ELISA using virus specific polyclonal antisera (Loewe, Germany). Samples collected earlier from the Punjab province and found infected with ZYMV were included as control (Malik *et al*., 2005).

Geminivirus Detection by PCR and Southern Hybridization: To test the presence of a begomovirus in symptomatic melon plants, total DNA was isolated from leaf samples using CTAB method and PCR was carried out using universal primers for begomoviruses. The primers used in PCR were Begomo1 (CCGTGCTGCTGCCCCCATTGTCCGCGTCAC) and Begomo2 (CTGCCACAACCATGGATTCACG CACAGGG) that are expected to produce a PCR product of about 1.1 kb. The other set of primers used in PCR consist of Begomo3 (GTTCCCCTGTGCGTGAATCCATGGTTGTGG) and Begomo4 (TTTTG TGACGCGGACAATGGGGGCAGCA) that are expected to produce a PCR product of about 1.7 kb. PCR was performed under standard conditions as described previously (Iram *et al*., 2005). Samples were also tested for the presence of begomoviruses using DNA A of *Tomato leaf curl New Delhi virus* (ToLCNDV) as general probe for begomoviruses (Padidam *et al*., 1995). Probe was prepared using Hexalabeling DNA labeling kit (Fermentas) and [α-^{32}P] dCTP (Amersham, U.K) Hybridization was carried out overnight at 65°C in a hybridization oven. Post hybridization washes were done at medium-stringency.

Results and Discussion

Out of 10 symptomatic samples tested for CMV, all were found positive and also same numbers were found positive for ZYMV. No positive reaction occurred in asymptomatic plants from the field or those grown under glasshouse conditions. As reported earlier samples collected from Punjab were found positive only for ZYMV (Malik *et al*., 2005). Present results indicate that both viruses (CMV and ZYMV) are present in dual infection.

For the detection of begomovirus total DNA was isolated from leaf samples and PCR was carried out using universal primers for begomoviruses. Amplification of PCR products of expected size 1.1 and 1.7 kb (Fig. 3) were obtained only from positive control whereas no amplification was obtained in all symptomatic samples collected from Mardan district. For further confirmation of these results, total DNA was also resolved in 1% agarose gels, blotted to nylon membranes and probed with radioactively-labeled probe of DNA A of ToLCNDV as general probe for begomoviruses. The blot was washed at medium stringency. The probes hybridize with positive control samples collected from symptomatic plants whereas did not hybridized with symptomatic samples from Mardan (data not shown). These results further confirmed the absence of begomovirus in symptomatic melon from Mardan.

Our screening for 3 viruses on symptomatic melon samples collected from Mardan District shows that plants were infected with CMV and ZYMV while all samples from Mardan were negative for begomoviruses. ZYMV has been reported previously from Pakistan (Khalid & Ahmad, 1997). However, CMV was not detected in those samples. In another recent study, cucurbit samples from NWFP were tested for the presence of 4 RNA viruses that did not include CMV (Ali *et al*., 2004). Symptoms observed on those plants were different from those reported here. Thus, our results are the first example of double infection of CMV and ZYMV from Pakistan associated with a severe outbreak on melon. The severe symptoms appear to result from synergistic interactions between ZYMV and CMV. We have ruled out a possible involvement of a begomovirus in this severe outbreak.

Synergistic interaction between potyviruses and CMV has been reported earlier where potyvirus supported higher level of CMV in dual infections (Grafton-Cardwell *et al*., 1996; Luis-Arteaga *et al*., 1998). This mixed infection causes considerable damage worldwide in severe epidemics in cucurbit fields. Double infection by ZYMV and CMV on zucchini squash (*Cucurbita pepo*) resulted in a synergistic effect where symptoms in doubly infected plants were more enhanced than plants singly infected by either virus (Fattoh, 2003). Wang *et al*., (2002) reported strong synergistic pathological responses in mixed infection with ZYMV and CMV in zucchini squash. This was attributed to increase in the level of accumulation of CMV positive strand RNA level. Examples of other synergistic combinations between potyvirus and viruses belonging to other genera are, *Potato virus* Y, *Tobacco vein mottling virus* or *Tobacco etch virus* together with the potexvirus *Potato virus* X in tobacco (Rochow & Ross, 1955; Damirdagh & Ross, 1967; Vance, 1991; Vance *et al*., 1995); ZYMV and either *Cucurbit aphid-borne yellow mosaic* in muskmelon (Bourdin & Lacoq, 1994) or the cucumovirus *Cucumber mosaic virus* in cucumber (Poolpol & Inouye, 1986).

Our results indicate that severe disease on melon in Mardan is caused by multiple infections of two RNA viruses that resulted in synergistic interactions in viruses belonging to two different groups.

Fig. 1. Symptoms on melons in commercial field in Mardan, NWFP. 1a. A commercial field in Mardan district showing widespread disease damage. 1b. Infected melon leaves showing enations on the upper side of leaf, vein thinking, leaf distortion and chlorosis.

Table 1. Melon viruses detected by DAS-ELISA in symptomatic samples from Mardan, NWFP compared to those from the Punjab province.

	Sample No.	CMV	ZYMV
Mardan Field 1	1	+	+
	2	+	+
Mardan Field 2	1	+	+
	2	+	+
Mardan Field 3	1	+	+
	2	+	+
Mardan Field 4	1	+	+
	2	+	+
Mardan Field 5	1	+	+
	2	+	+
Mardan Vehari, Punjab		-	+
Chichawatni, Punjab		-	+

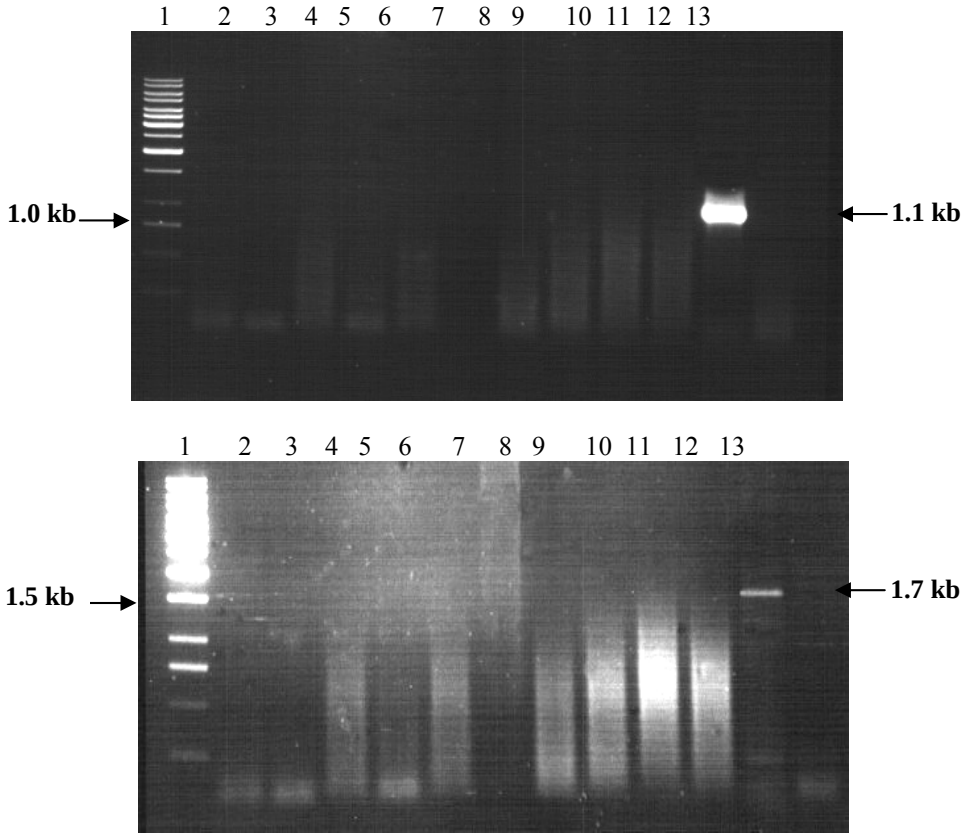

Fig. 2. PCR for the detection of begomovirus using universal primers for begomoviruses. Fig. 2a. PCR with universal primers for begomoviruses that produce a PCR product of about 1.1 kb. Lane 1 contain 1 kb ladder used as size markers where lane 2-11 represent melon samples where no PCR product was amplified. Lane 12 contain positive control where a tomato sample originating from Punjab was used as positive control whereas lane 13 represent negative control where distilled water was added in reaction mixture. Fig. 2b. Represents PCR with universal primers for begomoviruses that produce a PCR product of 1.7 kb. The sequence of samples in the lanes are same as given in Fig. 2a. Amplification product of expected size was obtained only positive control.

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